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Automated sample preparation and liquid chromatography-mass spectrometry for high throughput bioanalysis

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Automated Sample Preparation and Liquid Chromatography-Mass Spectrometry for High Throughput Bioanalysis

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Richard J. Grater

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This thesis is accepted and approved in partial fulfillment of the requirements for the Master of Science.

APRIL 24, 2003
Date

Thesis Advisor
(Advisor must sign in Blue Ink)

Co-Advisor

Chairman of Department

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I wish to express my sincere and deep appreciation and gratitude to my Major Thesis Advisors, Dr. James Roberts and Ned Heindel, and also my occupational advisor, Dr. Young Shin. They supplied me with their valuable advice, guidance, and support throughout the entire course of this investigation. Thank you so much for your comments, criticisms, and suggestions during the preparation of this thesis.

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Abstract:

The many roles mass spectrometry plays in the discovery and development of chemical entities by both the biotechnology and pharmaceutical industries are highlighted. Innovations in sample preparation for mass spectrometry have allowed accelerated the throughput of combinatorial chemistry and pharmacokinetic studies. Quantitative analysis of Liquid Chromatography coupled to a Tandem Mass Spectrometer (LC/MS/MS) has been used in this experiment for a dynamic range of 10 ng/mL to 2500 ng/mL in rat plasma of many analytes. The calibration curve in this experiment was generated using an automated method (Packard Multiprobe II) and compared to a manual method (prepared by an analyst). A student t test was used to determine if the automated vs. manual methods gave significantly different results.

A three day validation of compound A in rat plasma has been used to determine reproducibility, accuracy, and ruggedness of the assay using the Packard Multiprobe II robotic system. The samples were quantified utilizing Liquid Chromatography coupled to a Mass Spectrometer (LC/MS). The results from the validation were acceptable since the average within-run precision and average bias for all levels of quality control samples were less than 15 percent. The between-run precision and average bias for all validation runs were also less than 15 percent.

Introduction

High throughput screening and the application of combinatorial chemistry to compound discovery have changed the traditional serial process of lead optimization and identification that preceded investigations of the development of drugs. Recently, an incredible amount of new chemical entities have been synthesized in parallel using combinatorial chemistry. The compounds have been tested rapidly using high throughput screening, therefore, the synthesis of new discovery compounds is no longer the time limiting step. The new bottleneck is currently sample preparation of the thousands of pharmacokinetic, intrinsic clearance, protein binding, and other types of study test articles generated by the discovery or development groups in the pharmaceutical industry.

In order to cope with this new research and development trend in the pharmaceutical industry, several analytical instruments have been developed. The mass spectrometer is now one of the most critical tools for the entire drug discovery process using combinatorial chemistry and high throughput screening.

There has been a need to develop more automation techniques in conjunction with 96 well plates to be able to serve the demand of customers in the pharmaceutical industry. Statistical information and validations must be generated to assure that the newer techniques utilized are dependable.

History of LC/MS

Mass Spectrometry (MS) has been around for more than 80 years, but it has not impacted the drug discovery industry until recently. When mass spectrometry was introduced, it was narrowly focused on a set of gas-phase analytical techniques. A single sample was entered into the mass spectrometer via a vacuo lock and ionized in vacuo by bombardment with energized particles, usually electrons (electron bombardment ionization), neutral molecules (fast atom bombardment), or even through the transfer of energy (electric field ionization, laser desorption, or spark discharge).[1-2] The ionization in the gas phase was extremely difficult since mass spectrometry caused extremely high fragmentation of the analyte ion. The matrix would also cause an immense amount of interference with the analyte. Many scientists working with mass spectrometry encountered problems with selectivity, therefore, tandem MS was developed. A dramatic increase in the specificity came forth when Liquid Chromatography (LC) columns were added to separate samples into individual components before introduction into the mass spectrometer. Analytical columns were developed and used to separate compounds and endogenous peaks.

Only about 20% of all analytes of interest were analyzed by GC/MS.[1] The non-ionic compounds appeared to perform much better than ionic compounds, which kept GC/MS very limited for the pharmaceutical industry. Unfortunately, most of the drugs and metabolites are polar and ionic in nature.

One major difficulty was matching the condensed-phase system of the separating LC with the ultra high vacuum domain of the mass spectrometer. The three problems that essentially had to be solved were the volatility of the solvent (used in the LC), the flow rate of the mobile phase and the high polarity of the compounds. The issues that were the most significant in LC/MS were analyte stability, ionizability, and volatility.

A more "primitive" approach that scientists used for LC/MS a moving belt interface to continually deposit drops of mobile phase effluent from the LC experiment onto a tiny conveyer belt.[1] After the liquid vaporized, the residue was transferred to the ion source and electron impact was utilized. Electron impact appears to have an extremely poor response time, a decrease in resolution, and high carryover. Other types of interfaces tried included a pneumatic nebulizer, continuous flow fast atom bombardment, and particle beam ionization. The interfaces did have an increase in performance, but fast atom bombardment was not able to handle reasonable flow rates. Unfortunately, particle ionization was performed at high temperatures, which caused thermal instability of the compounds. Scientists learned a new lesson whenever new techniques failed.

One of the most significant improvements was discovered when the solvent was eliminated and the nonvolatile analytes were ionized at atmospheric pressure. From this liquid-based ionization strategy, Atmospheric Pressure Ionization (API) mass spectrometry was born.

One could now use either thermospray (very soft ionization and strong heating) or electrospray (soft ionization, high voltage, mild heating).[1] These two techniques were complementary in applicability and performance. For compounds that were less polar APCI (Atmospheric Pressure Chemical Ionization) could be used. A corona discharge needle was required for APCI source change. The needle assists in the transfer of a charge to the solvent and could be used at an increased flow rate such as 1 mL/min.

Virtually everything on the modern LC/MS is controlled by computer. After connecting the LC with the mass spectrometer, scientists could now enjoy tremendous selectivity, sensitivity, obtain both qualitative and quantitative data, all applicable to many small molecules of pharmaceutical interest.

With the extreme selectivity and the separation power of the LC, now mass spectrometrists could now analyze a single sample containing up to twenty analytes. Molecular weights of compounds could be determined by infusing a solvent containing the analytes.

A mass spectrometer is capable of the following tasks: separation, formation, rearrangement of molecular ions and detection of molecular ions based on the mass to charge ratio (m/z). The compartments that make up a mass spectrometer are a sample inlet, an ionization source, a mass analyzer, a detector, and a data processor.

The ion is either positively or negatively charged depending on the functional groups of the molecule and the ionization mode. For example, positively charged

molecules generally had amides and amines in their structures.[1] Negatively charged ions frequently have carboxylic acid groups and/or hydroxyl functional groups.[1] A molecule may be singly or multiply charged depending on its structure. If a compound has a molecular weight of 522 and it only has one positive charged, then the molecular ion $[M+H]^+$ will be 523. Conversely, if this particular molecule had a single negative charge the molecular ion $[M-H]^-$ on the mass spectrum would be 521. A double positive charge on the same molecule would have a molecular ion of (m/z) molecule + H^+ + H^+ / 2 = 262. There would be two protons added to the molecule, and the m/z ratio is cut in half by the two charges.

The resolution of the mass spectrometer is defined qualitatively as its ability to discriminate between adjacent ions in a spectrum.[1] The resolution is defined as the function of molecular weight and is given by the following equation:

$$\text{Resolution} = m / \Delta m$$

where m = the molecular weight

Δm = peak width of the ion at half-height, at a given percentage valley between the adjacent ions.

Resolution is increased when the peak width decreases for a given m, or as m is larger without a concurrent increase in peak width.

Combinatorial chemistry and mass spectrometry

During the past several years, combinatorial chemistry has been a key technology for accelerating the discovery of novel therapeutic agents in the agrochemical, biotechnology, and the pharmaceutical industries.[3] Combinatorial chemistry was first introduced by the seminal work of Merrifield[4], involving solid-phase synthesis protocols were utilized to create small mixtures of peptides on solid supports. This early work was the foundation of peptide libraries and combinatorial chemistry. Combinatorial chemistry has essentially become an integral component of nearly all drug discovery efforts.[5-8]

Mass spectrometry is one of the most useful instruments in the advancement of combinatorial chemistry through the optimization of library synthesis and reaction monitoring, assessment of library compound quality, and in the bioaffinity and screening pharmaceutical properties of the above. The major benefits of using mass spectrometry are the high sensitivity, specificity of detection, and short analysis time.[9]

One of the simplest ways of sample introduction to a mass spectrometer is called Flow Injection Analysis (FIA). Minimizing carryover using FIA/MS of combinatorial compounds was accomplished by Richmond and Goerlach.[10-12] The samples were sorted by maximizing the molecular weight of each adjacent sample injected. Carryover was determined to be approximately 0.01 % and the cycle time was less than one minute per sample.

High throughput characterization of combinatorial libraries using parallel systems

Parallel injection may further increase FIA/MS throughput. Parallel injection consists of a four or eight channel sprayer head (multiplexed electrospray inlet) that directs the spray directly into an orthogonal acceleration Time of Flight (TOF) MS or triple quadrupole MS. The sprayer streams are introduced into the MS simultaneously, however, these streams must be sampled one at a time to minimize cross contamination between the channels. The diagram for the four channel sprayer head is in Figure 1.

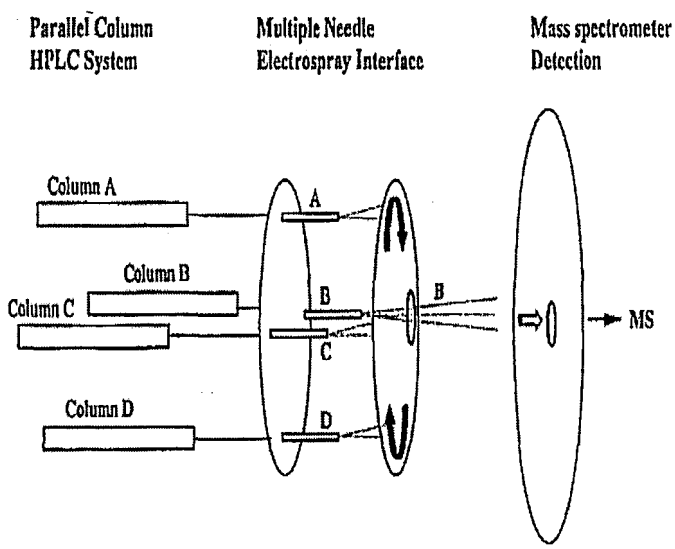


Figure 1. Parallel LC/MS with four HPLC columns interfaced simultaneously to one mass spectrophotometer, which provides an increase in throughput of LC/MS. This example demonstrates eluent from each HPLC column electrosprayed into a single ion source, but only one HPLC stream is sampled at a time by means of a rotating plate shown aligned with column "B", thus the other sprays are blocked by the plate at this particular instant. (Based on Reference Shin et al. [13], 2001.)

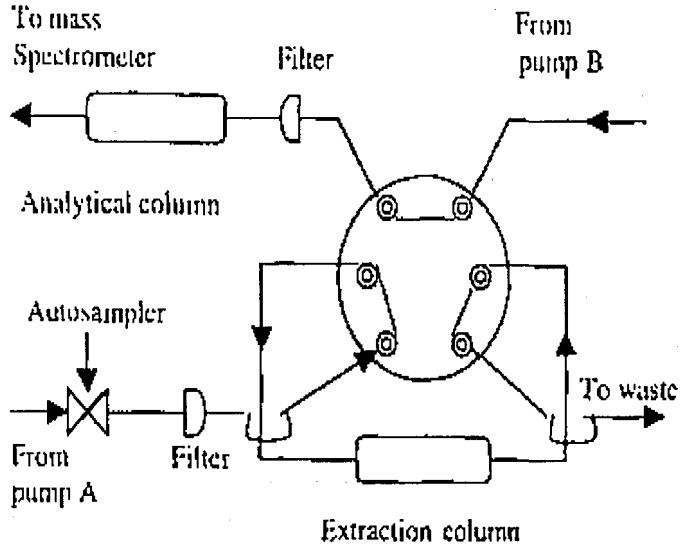
Each stream of liquid was sampled for 0.1 seconds and individual mass spectra for each analytical column were acquired in four separate data files that were synchronized with the spray being sampled.[14] Four and eight channel sources with the same orthogonal acceleration TOF instrument were developed; cycle times were 0.6 s and 1.2 s, respectively.[15] Using an eight channel source with a 5 minute HPLC High Performance Liquid Chromatography (HPLC) gradient elution, a 96-well microtiter plate could be analyzed in 60 minutes.

Turbulent Flow Chromatography – Mass Spectrometry (TFC-MS)

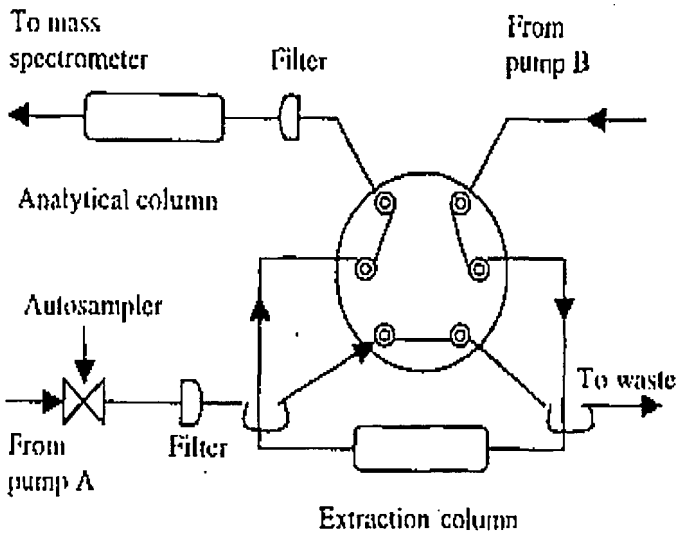
Rapid LC/MS cycle times remain important for MS analysis of large sample libraries.[16] One of the most useful, rapid, and more widely used on-line LC/MS techniques is Turbulent Flow Chromatography (TFC).

In TFC as many as four separate systems are coupled to a single MS. Each unit is composed of two separate LCs, one of which elutes solvent to the turbulent column and the other pump elutes solvent to the analytical column. The turbulent pump initially uses a high flow rate (5 mL of aqueous buffer), which flows through a turbulent column packed with a narrow internal diameter and large size particles (50μm). The pressures are more manageable (700-1200 psi) for direct-injection techniques with such large particles.[16] A size exclusion type of separation mechanism is observed with turbulent flow. Macromolecules do not rapidly diffuse into the particles pores and elute without retention, whereas small particles do diffuse and are

retained on the column. Virtually all of the polar endogenous particles are washed off the turbulent column by a rapid clean-up step. The compounds of interest are not affected by the rapid rinsing and remain on the column. An organic solvent such as acetonitrile is used to elute the compounds from the turbulent column to the analytical column. Both the organic and aqueous solvents will contain a low concentration of acid to assist in the ionization of the compounds. The compounds are retained on the analytical column during a gradient program change from 50/50 organic/aqueous phase to 100% organic phase to separate and elute the compounds into the MS. As the mass spectra are obtained from the first sample, the next unit is injected onto the turbulent column. Each of the four units is sampled in turn for a 1.5 minute MS acquisition time, yielding a total of slightly over six minutes for each cycle. Figure 2 shows the set up of the TFC-MS.



Configuration (a): Loading sample, extraction and equilibration



Configuration (b): Elution

Figure 2. A schematic presentation of turbulent flow on-line extraction LC/MS/MS. Pump A represents the aqueous mobile phase and Pump B represents the organic solvent. (Based on Reference Jemal, [16], 2000.)

The application details and principles of turbulent flow on-line extractions are found in a number of articles.[17-18]

Capillary Liquid Chromatography

As more potent drugs are discovered in the biotech and pharmaceutical industry, the need to achieve better sensitivity will be a focal point. Increasing sensitivity would also require less volume of blood or other matrices from rodent animals or pediatric patients in order to allow several serial bleeds on each animal. After the LC conditions, MS parameters, and chromatographic peak efficiency have been optimized for a given assay there is little one could improve. One possibility to increase sensitivity involves capillary LC columns for bioanalytical LC/MS/MS methods.[19-21] A 100-fold increase in the analyte peak concentration at the detector can theoretically be achieved by simply reducing the diameter of the LC column from 2mm (commonly used for LC/MS), to a capillary column dimension of 0.2mm. Unfortunately, high backpressures are observed with capillary columns due to the small internal diameter of the columns. The amount of sample that may be injected is limited with a capillary column. It remains to be seen if rugged high throughput capillary LC/MS/MS bioanalytical methods with short run times can be generated and routinely used.

Robotics for sample preparation

Another method for increasing analysis and screening throughput of combinatorial libraries is the use of automated liquid handling systems. Currently, the most frequently used robotic modules for 96-well vials are the Packard Multiprobe (Packard Instruments, Meriden, CT, USA), Tecan (Durham, NC, USA), and Tomtec Quadra (Tomtec, Hamden, CT, USA).[22] The Tomtec has been successfully validated by measuring concentrations of compounds in plasma[23], blood[24], and urine.[25]

The Packard Multiprobe liquid handling system has many strengths for offline Solid Phase Extraction (SPE) methods involving plasma[26] and serum[27]. This unit and the Tecan can be programmed to initially aliquot samples from the vials to the 96-well blocks, then buffer and an internal standard. The amount of time and labor is significantly reduced. Either the replacement of disposable pipet tips or several washing steps need to be inserted into the sequence to alleviate carryover. Caution must also be taken to assure that endogenous protein clots do not clog the probes. This may cause inaccurate transfer from the reservoirs to the 96-well blocks.

The most common types of extraction techniques are: Liquid-Liquid Extractions (LLE), Solid-Phase Extraction (SPE), and Protein Precipitation (PPT). All robotics systems have been successfully used for these types of extractions. Based on a series of experiments, King and colleagues has determined the order of ESI response suppression is PPT>SPE>LLE, with the least amount of analyte ion loss

for LLE.[28] Before 1999, LLE was not very prevalent in the pharmaceutical industry. There has been a dramatic increase in the number of articles published in this methodology since then.[29-31] An automated sample preparation method based on a 96 well plate platform has been developed for high throughput analysis using LC/MS/MS. A three day validation of accuracy and ruggedness was also evaluated. The usefulness of this method is discussed in some detail.

Experimental Methods

Chemicals

Compounds A, B, and the internal standard were GlaxoSmithKline Pharmaceuticals discovery compounds. Their actual identity is not important for testing the validity of the tested methods.

Acetonitrile (ACN) – HPLC grade	EMD Chemicals Inc. (Gibbstown, NJ)
Ammonium formate	Fisher Scientific (Fair Lawn, NJ)
Formic acid – reagent grade	Sigma Chemical Co. (St. Louis, MO)
Water, HPLC grade, Milli-Q system	Millipore Co. (Bedford, MA)
Rat Plasma – heparinized	Biological Specialties (Colmar, PA)

10 mM ammonium formate, pH 3.0 preparation

To prepare this buffer , 0.63 g. of ammonium formate, was dissolved in 1.00 L of Millipore water and adjusted to pH 3.0 with formic acid. The buffer was then filtered through a 0.2 μm filter before use.

Equipment

HPLC

A quaternary solvent pumping system including two 1100 HPLC pumps from Agilent Technologies (Palo Alto, CA) was used to deliver mobile phase to a 50mm X 3mm C18, 5μm particle size HPLC column from Phenomenex Inc. (Torrance, CA) preceded by a 0.5 μm pre-column filter. All injections were made by a CTC Analytics Pal autosampler (Zwingen, Switzerland).

MS/MS

An API-4000 triple quadrupole mass spectrometer from MDS Sciex Instruments (Concord, Ontario, Canada) was coupled to the HPLC via a Turbo IonSpray interface. Data acquisition, display, and automatic data processing were performed using the Analyst™ software version 1.2, supplied by MDS Sciex Instruments.

Automated Liquid Handling System

A Multiprobe II HT EX automated liquid handling system from Packard (Wellesley, MA) was used for sample and standard preparation. The multiprobe did

not use any disposable pipet tips but instead used fixed tips with a flush wash between liquid transfer. The multiprobe II used eight probes or arms to dispense and aspirate reagents. The cycle time for the automated method was approximately eleven minutes to prepare the standard curve or quality control samples. Appropriate methods were prepared separately for the standard curve and the quality control samples. All probes were sufficiently rinsed with Milli-Q water after each aspiration and delivery of reagents.

Other equipment and materials

Eppendorf micro-centrifuge, model 5413	Brinkmann Instruments, Inc. (Westbury, NY)
Eppendorf 1.5mL polypropylene tubes	Brinkmann Instruments, Inc. (Westbury, NY)
96 well plates and deep tubes	MTX Labs Inc. (Vienna, Virginia)
MultiTube vortexer	Baxter Scientific Products (McGraw Park, IL)
Eppendorf pipettes	Brinkmann Instruments Inc. (Westbury, NY)

LC and MS conditions used

Liquid Chromatography Conditions

LC used:	Agilent quaternary 1100 HPLC pump (isocratic)
Mobile Phase Composition:	72/28 acetonitrile/10mM ammonium formate, pH 3.0
Flow Rate:	450 µL/ minute (no split)
Column used:	Phenomenex C18, 5µm particle size, 50 X 3mm (length X internal diameter)
Injection volume:	10 µL
Acquisition time:	0.9 minute

MS Parameters

Mass Spectrometer used:	MDS Sciex API-4000 Turbo Ionspray
Scan Type:	MRM (Multiple Reaction Monitoring)
Resolution for Q1 and Q3:	Unit Mass
Pause Time:	5.0 ms
Polarity Mode:	negative
Temperature:	450 °C
Ion Spray Voltage:	- 4200
All compounds (Dwell time):	100 ms

Compound	Precursor Ion (m/z)	Product Ions (m/z)	DP	CE	CXP
A	415	242	-46	-16	-13
B	459	398	-82	-28	-11
Internal Std.	328	267	-70	-30	-15

DP: Declustering Potential **CE:** Collision Energy **CXP:** Collision Exit Potential

Compounds A, B, and the internal standard all had a single negative charge and had molecular weights of 416, 460, and 329, respectively.

Results and Discussion

One of the most important steps for bioanalysis is standard and sample preparation. Utilization of automated liquid handling systems should increase efficiency, minimize error, and free analyst time. Two assays were performed in this experiment. The first assay was used to compare automated vs. manual sample preparation to make sure that the results were indistinguishable within the 95% confidence level. The second experiment was a three day validation on the Packard

Multiprobe II liquid handling system which included standards and quality control samples. Utilization of automated liquid handling systems should increase efficiency, minimize error, and is less time consuming compared to manual sample preparation.

Development of LC/MS/MS Analysis

To determine the MRM (Multi reaction monitoring) transitions, a Q1 full scan was performed to determine the precursor ions. After the precursor ions were determined, the declustering potentials were optimized for each analyte. A separate product ion scan of each precursor ion was used to select the product ions. The collision energy and collision exit potential were optimized for each analyte. The Q1 full scan and product ion scan for compound A are shown in Figures 3 and 4, respectively. The Q1 full scan and product ion scans for compound B are shown in Figures 5 and 6, respectively. The Q1 full scan and product ion scan for the internal standard are shown in Figures 7 and 8, respectively. All compounds were infused separately in 80/20 acetonitrile / 10 mM ammonium formate (pH 3.0) at a flow rate of 450 μL/min.

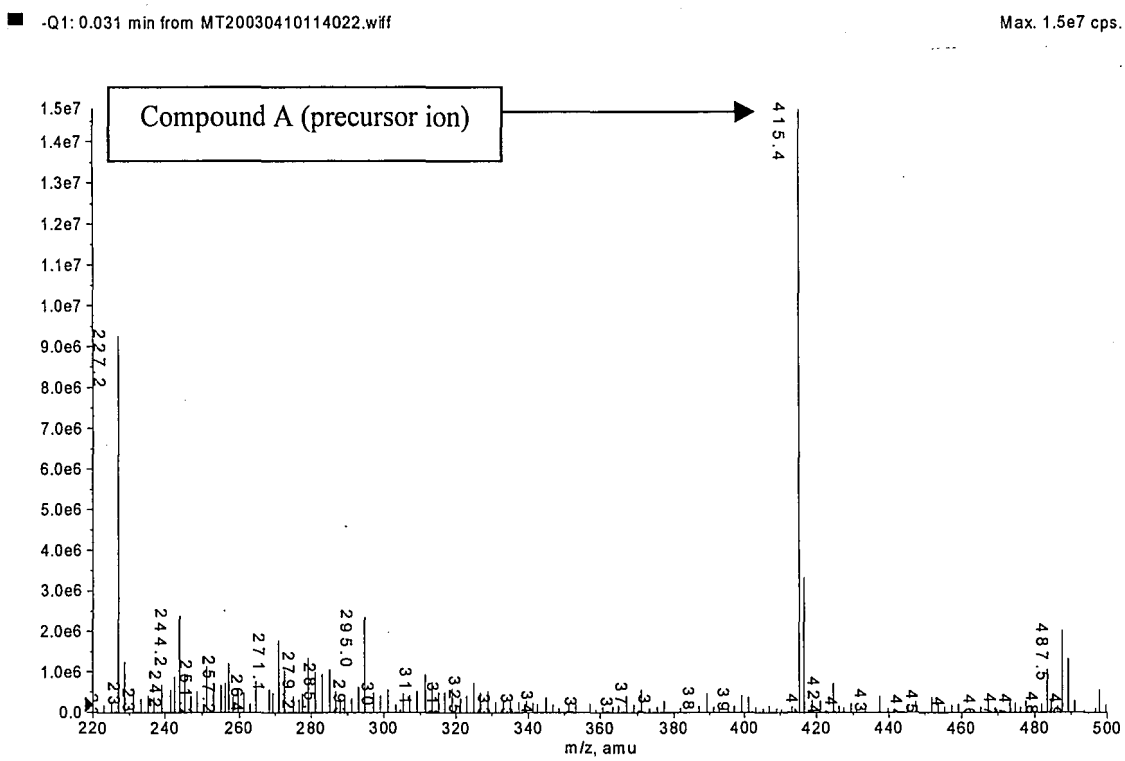


Figure 3. The Q1 full scan of Compound A at 100 ng/mL.

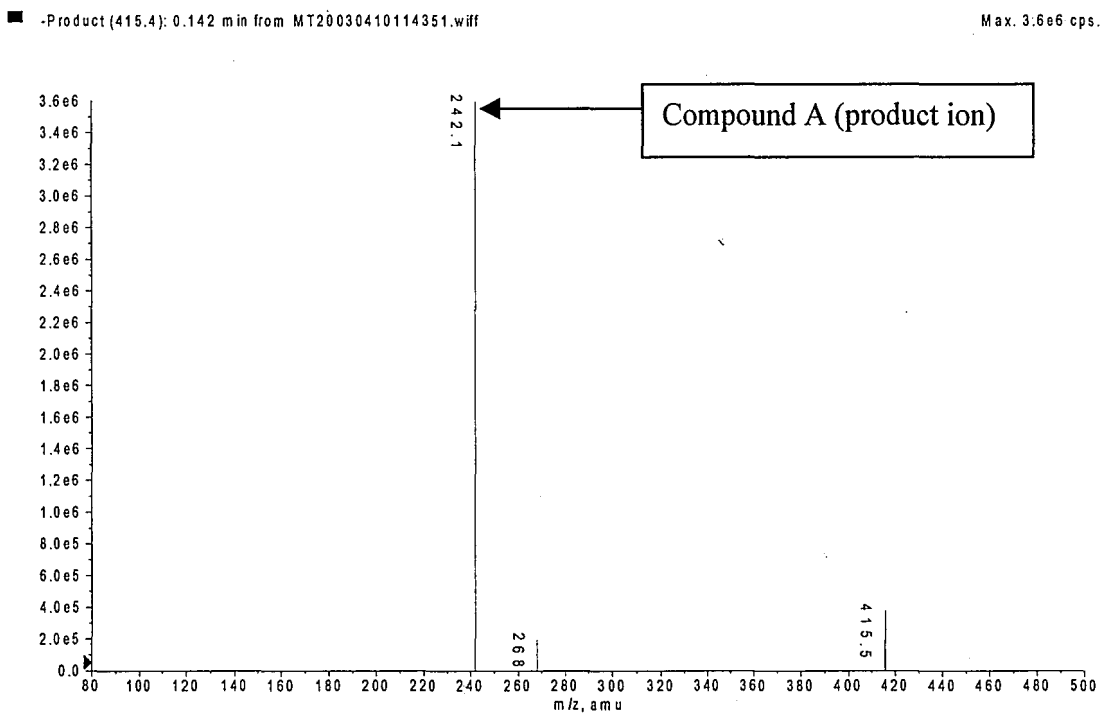


Figure 4. Product ion scan of Compound A at collision energy of –16eV.

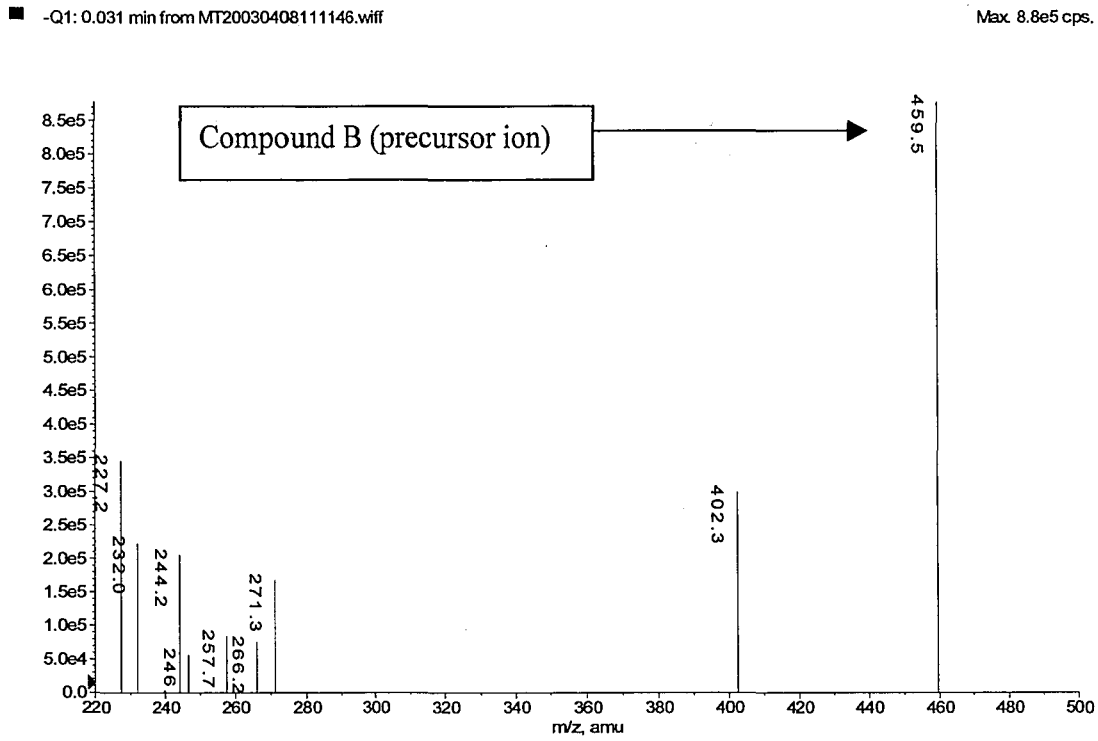


Figure 5. The Q1 full scan of Compound B at 100 ng/mL.

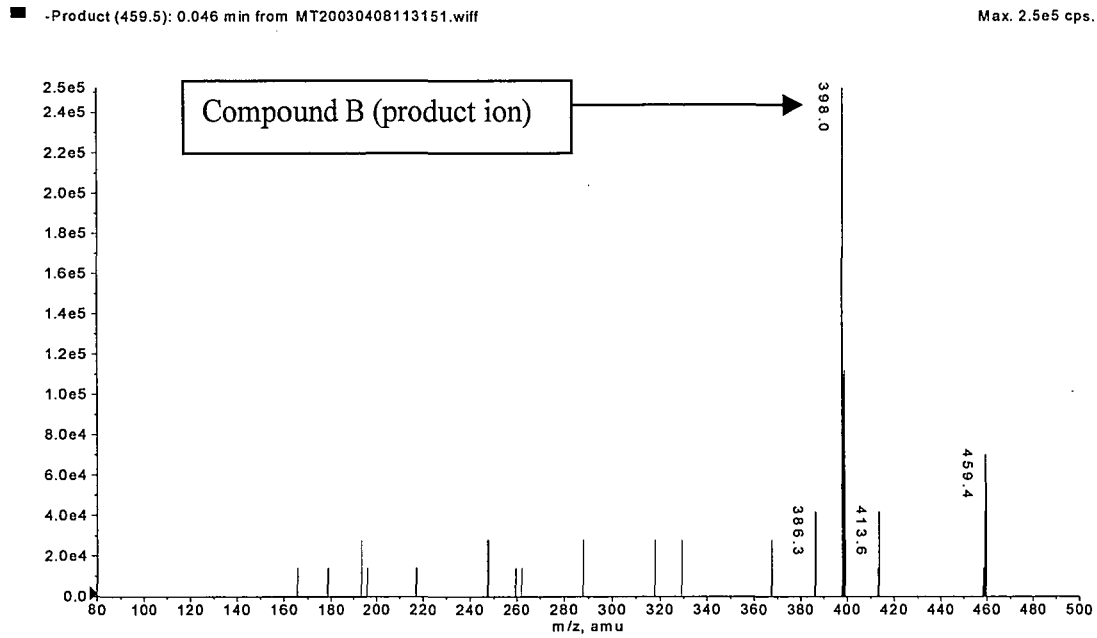


Figure 6. The product ion scan of Compound B at a collision energy of –26 eV.

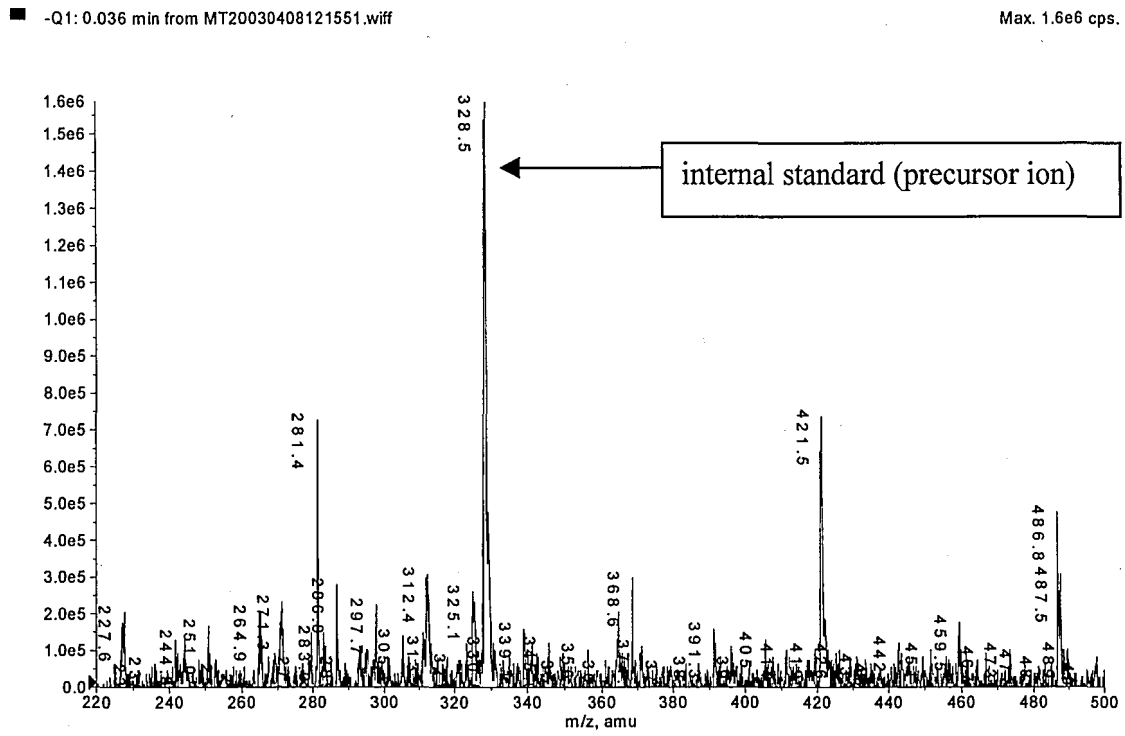


Figure 7. The Q1 full scan of the internal standard at 50 ng/mL.

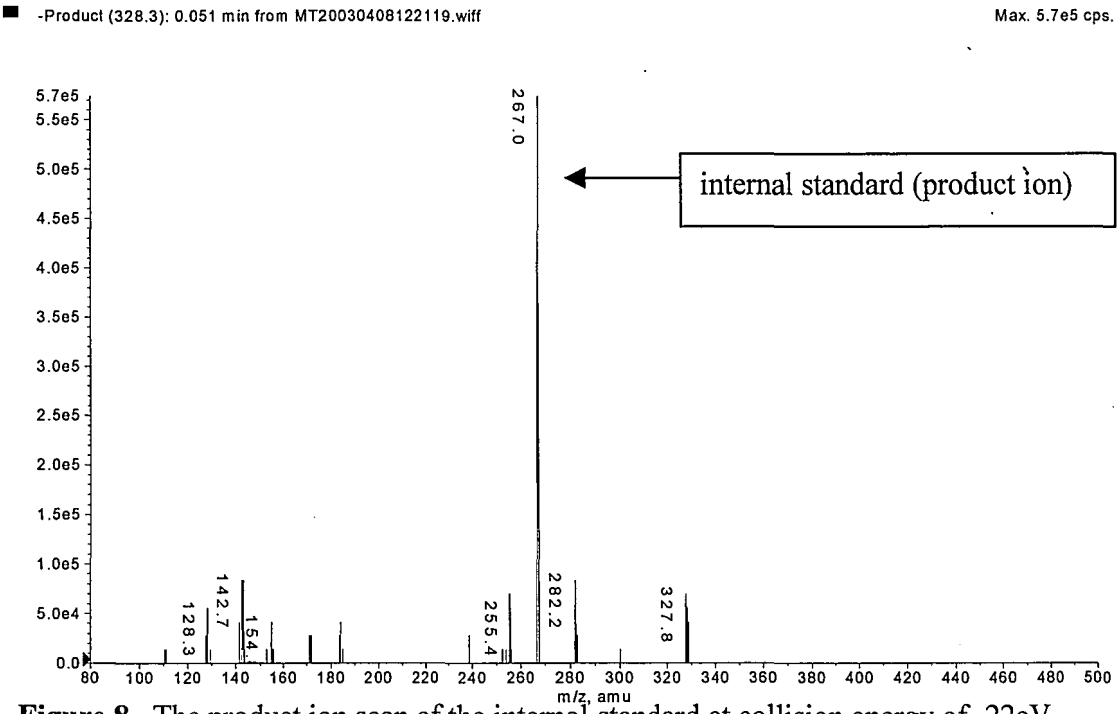


Figure 8. The product ion scan of the internal standard at collision energy of -22eV.

Automation vs. Manual Sample Preparation

The comparison of the two standard curves consisted of preparing two calibration curves from 10 ng/mL to 2500 ng/mL in heparinized rat plasma on the same day separately by both manual and automated methods. The samples were quantitated by using an Agilent quaternary 1100 HPLC pump coupled to a Sciex API-4000 MS/MS. Electrospray LC/MS/MS was chosen for this experiment because of the polar nature of the compounds, the high degree of selectivity, sensitivity, and rapid analysis. A photograph of the Packard Multiprobe II are in Illustration #1. A magnified picture of the probes, 96 well plate positions, and wash stations are shown in Illustration #2.

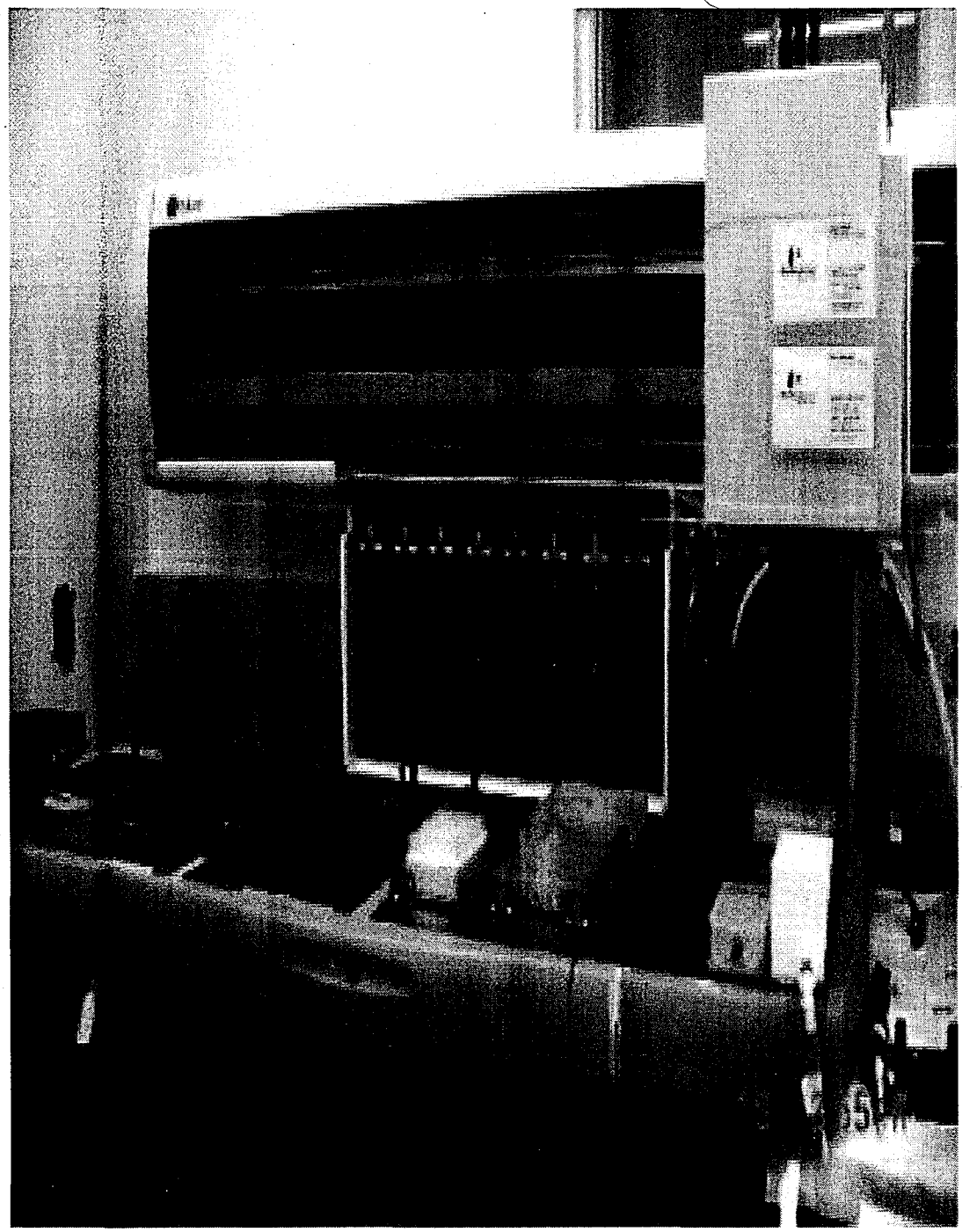


Illustration 1. A photograph of the Packard Multiprobe II Liquid Handling System consisting of eight samples aspirating and dispensing arms.

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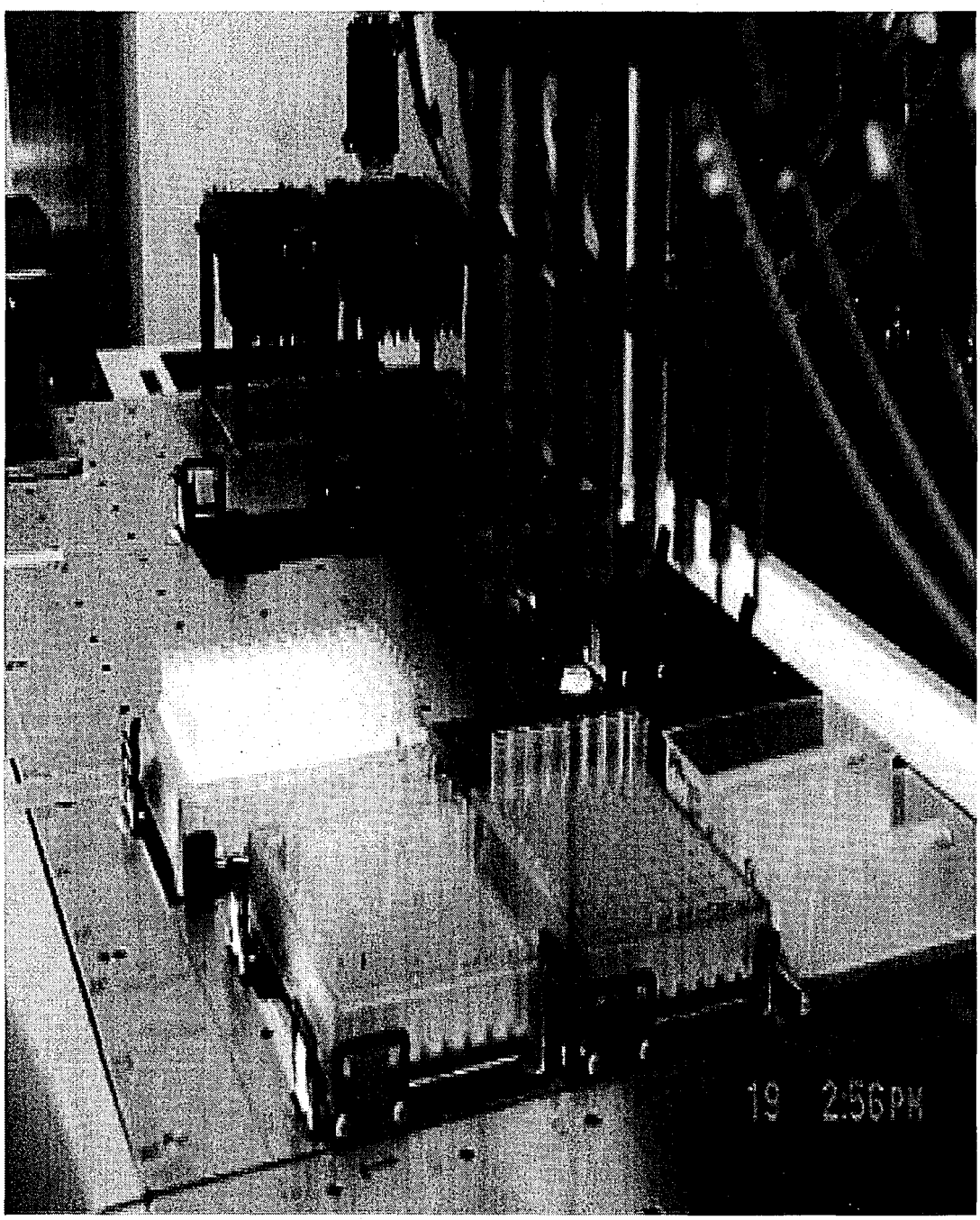

Illustration 2. A magnified picture of the Multiprobe II showing the 96 well tube racks (bottom left), eight probes (center), and the solvent reservoirs (right side).

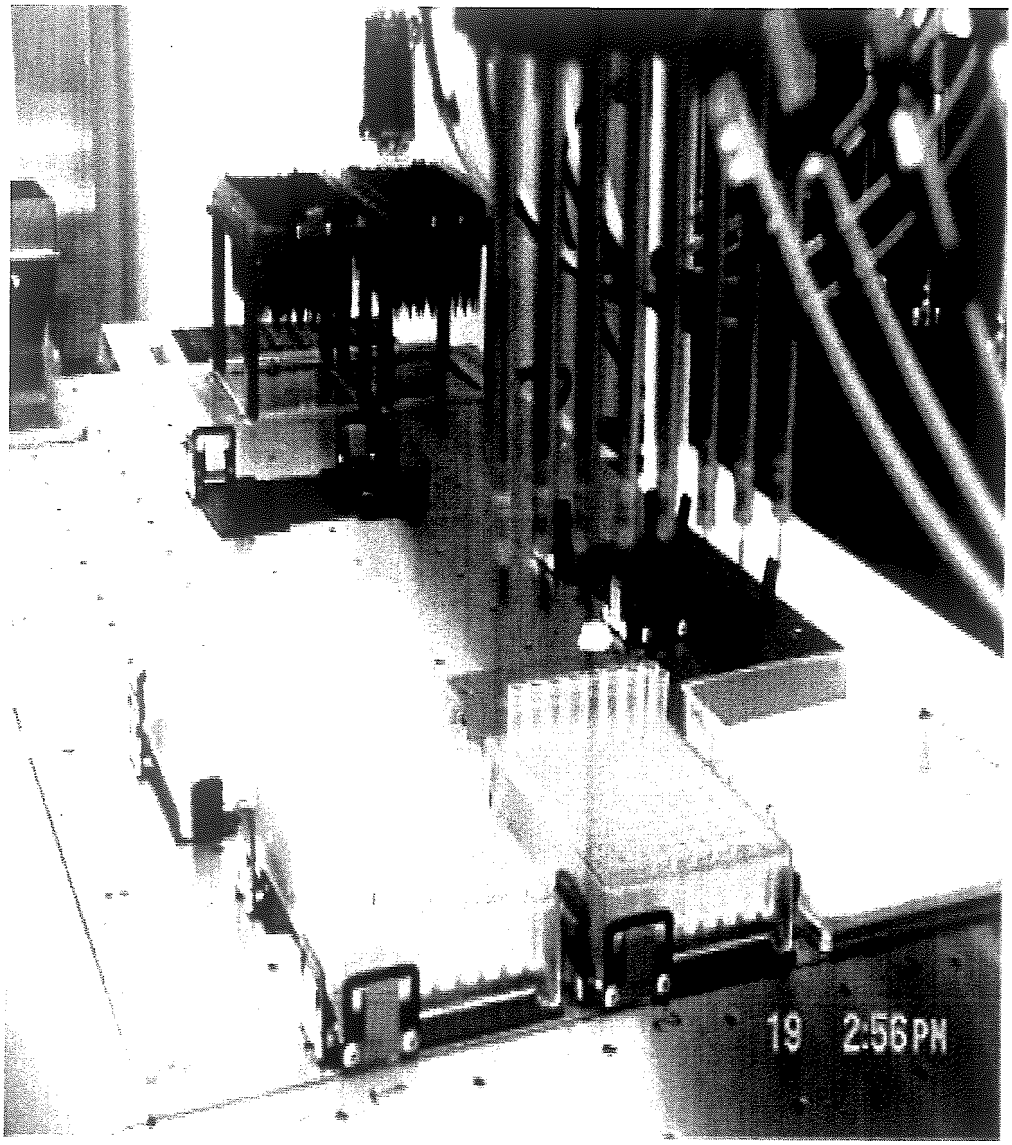

Illustration 2. A magnified picture of the Multiprobe II showing the 96 well tube racks (bottom left), eight probes (center), and the solvent reservoirs (right side).

Each 96 well plate, solvent reservoir, and wash station has a designated position on the multiprobe layout (i.e. E10). The designated position for each accessory must match the program method, otherwise the probes will be directed to the wrong positions. The multiprobe is capable of preparing standards and quality control samples using two separate methods.

Table 1 illustrates how the automated and manual calibration curves were prepared in rat plasma. Both calibration curves were prepared in 96 well tubes. Both the manual and automated standard curves were prepared within an hour of each other.

µg/mL	Solution used	compounds	stock volumes	diluent volumes
100	1 mg/mL	A and B	40 of each	320
20	100		80	320
2	20		40	360
0.2	2		40	360

standard curve conc. (ng/mL)	Std. Used	volumes of solution	volumes of rat plasma
2500	100	7.5	292.5
1000	20	15	285
500	20	7.5	292.5
250	20	7.5	592.5
100	2	15	285
50	2	7.5	292.5
25	2	7.5	592.5
10	0.2	15	285

Table 1. Manual and Automated Standard curve preparation for Compounds A and B.
Note: 50/50 acetonitrile/water was used as the diluent. All volumes are in µL.

After the preparation of the standard curves, 50 uL of each standard was aliquoted in duplicate into separate tubes and 100 uL of internal standard (IS) in acetonitrile was added and the solution mixed by vortex. Each standard was centrifuged for ten minutes at 4000 rpm

Standard curves were generated for both the manual and automated preparation methods for Compounds A and B. The calibration curves for Compounds A and B are shown in Figures 9 and 10, respectively. A duplicate set of calibration standards was analyzed with every concentration. A weighted method (1/x) was used to construct each calibration plot for the peak area ratio of analyte to internal standard versus analyte concentration. The calibration curves did appear to be linear. There were no standard curve points deleted from Figures 9 and 10.

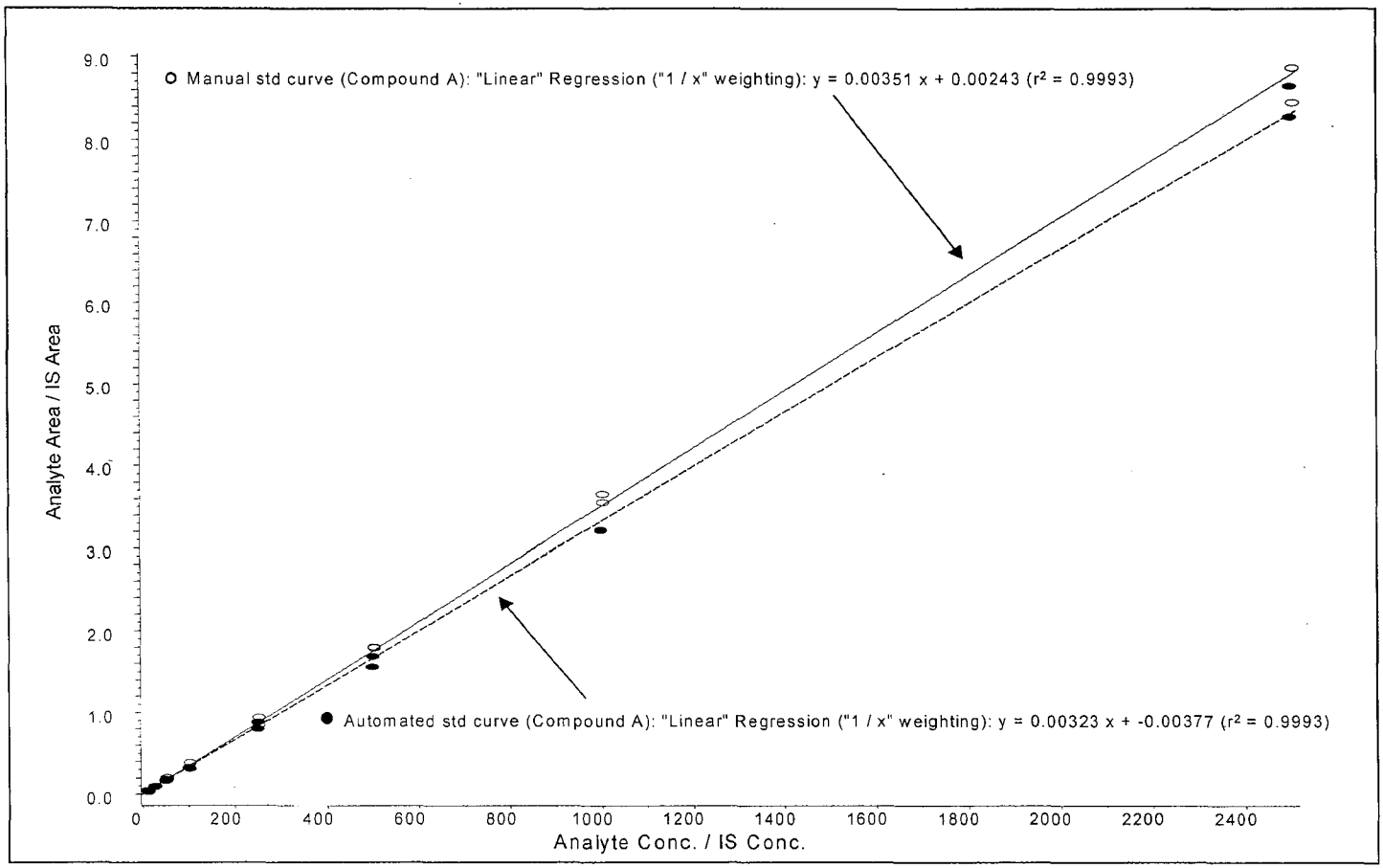

Figure 9. Calibration curves of Compound A with both the manual and automated methods are included in this figure. The manual standard points are hollow points and the automated standard points are solid points.

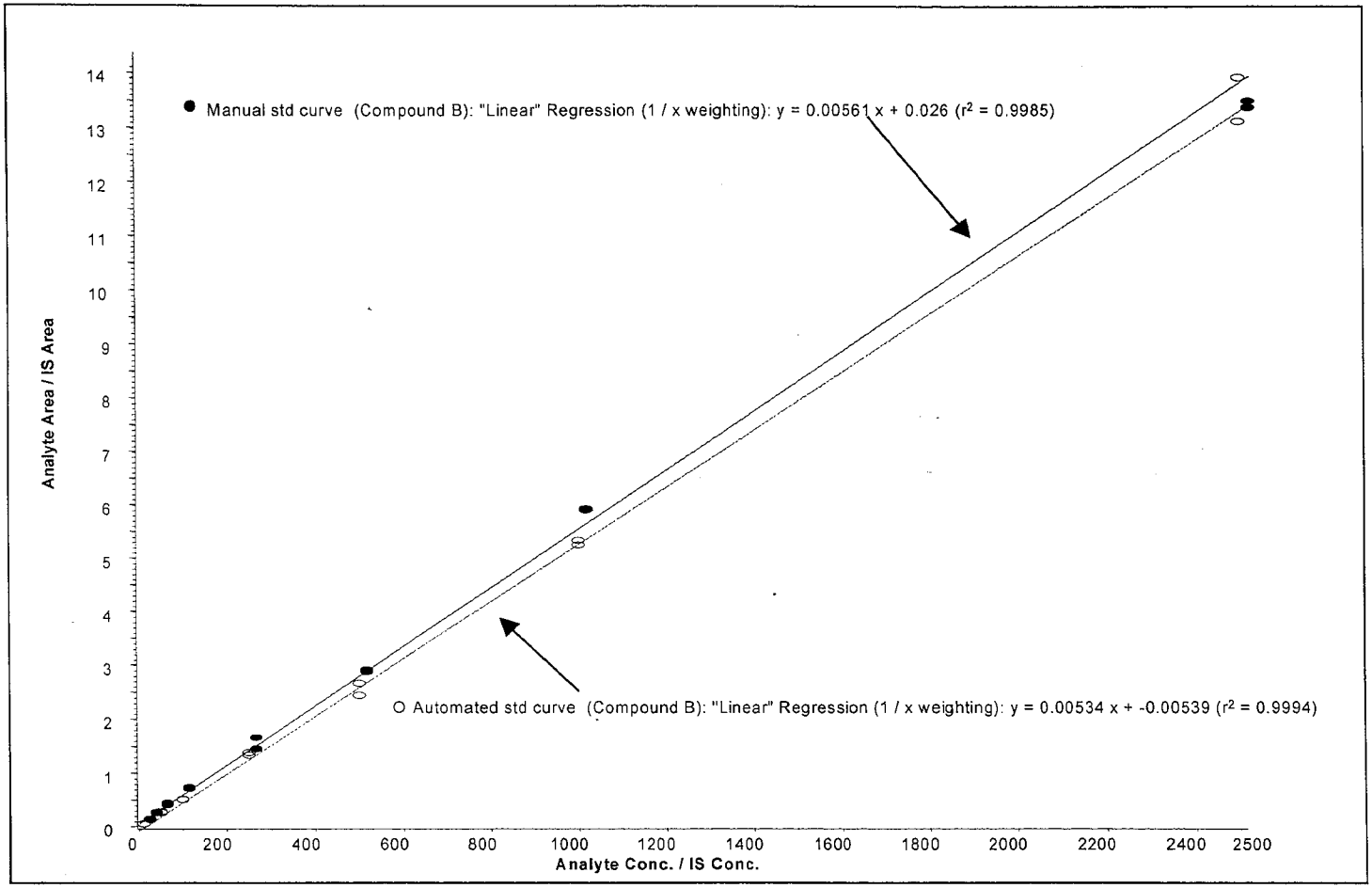

Figure 10. Calibration curves of Compound B with both the manual and automated methods are included in this Figure. The manual standards have solid points and the automated standards have hollow points.

In Figures 9 and 10 the manual sample preparation curves was slightly higher than the automated curves. The systematic error may have occurred from the manual pipets having a tolerance +/- 3%. The volumes may have been slightly greater than expected. The rat plasma used was heparinized. The student's t-Test was used to compare the two standard curves to determine if the data were within the acceptable limit of a 95% confidence level. The case 3 comparison of means with Student t test was used to calculate the t statistic.[33] There were two key calculations used in this experiment for each of the compounds. The first equation was to determine the S_d or the standard deviation of the individual experiment as shown below:

$$S_d = \left[\frac{\Sigma(di - d_{avg.})^2}{n-1}\right]^{1/2}$$

di – differences of the concentrations from each method at each level.
d_{avg} – the average difference between the methods throughout all data points.
n – is the number of pairs of data (eight in this experiment)

$$t_{calculated} = \frac{davg}{s_d} * (n)^{1/2}$$

$t_{calculated}$ – the experimentally determined value for the t-test.

Table 2 and Table 3 display the standard curve individual data points from compounds A and B, respectively. The calculations are also included below the tables.

In this laboratory, there are usually between two and sixteen compounds in one study with multiple subjects. Figure 11 illustrates a flowchart of the sequence for

analyzing compounds, samples, and standards to meet the required short turnaround times.

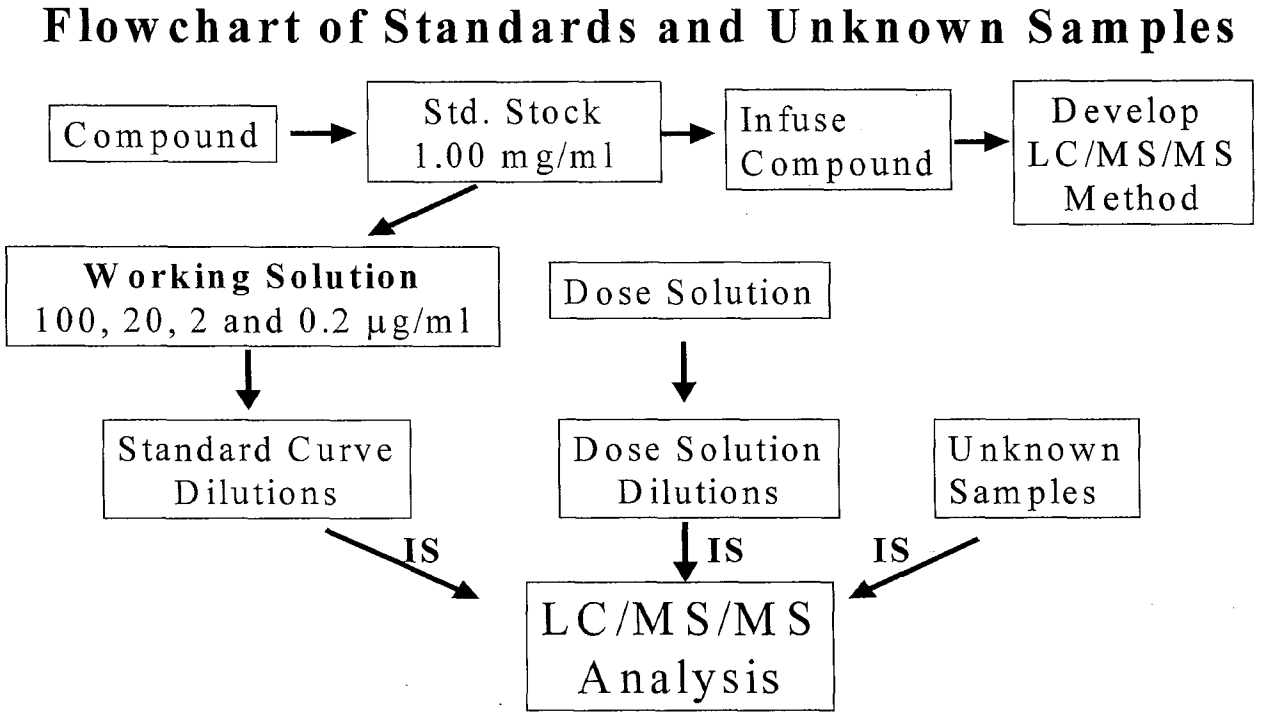

Figure 11. Flowchart of the process for analyzing multiple compounds. Step one – Prepare a 1.00 mg/mL stock solution for each compound and infuse/determine parameters on the LC/MS/MS. Step two- create the standard curve from the four working solutions and dilute the dose solutions. Add Internal Standard (IS) to the standard curve samples, diluted dose solutions, and the unknown samples.

Compound A nominal conc.	*Automated* rep #1	rep #2	mean	Compound A nominal conc.	*Manual* rep #1	rep #2	mean	difference
10	10.1	11.6	10.9	10	12.2	10.6	11.4	0.5
25	25.0	26.1	25.5	25	30.3	30.7	30.5	5.0
50	47.3	49.4	48.4	50	62.4	57.3	59.9	11.5
100	96.5	94.7	95.6	100	118.5	96.4	107.5	11.9
250	264.9	238.9	251.9	250	292.6	263.7	278.2	26.3
500	507.5	468.1	487.8	500	555.3	558.6	557.0	69.2
1000	966.6	969.0	967.8	1000	1132.6	1102.0	1117.3	149.5
2500	2490.6	2603.7	2547.2	2500	2745.1	2614.5	2679.8	132.7
							average d =	50.8

(D-D)	$(D-D)^2$		
-50.3	2525.3		
-45.8	2100.6		
-39.3	1544.7		
-39.0	1517.3		
-24.6	602.8		
18.3	336.6		
98.7	9741.2		
81.8	6699.0		
SUM =	3581.1	**S_d =**	59.8
		$t_{calculated}$ =	**2.246**

Table 2. Comparison of the manual vs. automated calibration curve preparation using the student's t-test determination of Compound A. At seven degrees of freedom for 95% confidence level the $t_{calculated}$ and t_{table} were determined to be 2.246 and 2.365, respectively. In this case, the $t_{calculated} < t_{table}$, thus the data are within the 95% confidence level. On the other hand, if $t_{calculated} > t_{table}$ then the two data sets would have been considered different from one another with 95% confidence.

Compound B nominal conc.	*Automated* rep #1	rep #2	mean	Compound B nominal conc.	*Manual* rep #1	rep #2	mean	difference
10	9.6	11.0	10.3	10	12.2	12.0	12.1	1.8
25	24.9	25.5	25.2	25	33.3	34.9	34.1	8.9
50	49.1	53.3	51.2	50	69.9	61.9	65.9	14.7
100	95.0	96.7	95.9	100	125.6	119.1	122.4	26.5
250	258.9	250.5	254.7	250	301.0	261.1	281.1	26.4
500	498.5	457.5	478.0	500	532.2	540.0	536.1	58.1
1000	983.8	999.7	991.8	1000	1108.8	1115.1	1112.0	120.2
2500	2453.1	2603.0	2528.1	2500	2550.2	2527.3	2538.8	10.7
							average d =	33.4

(D-D)	**$(D-D)^2$**		
-31.6	999.0		
-24.5	600.6		
-18.7	349.9		
-6.9	47.7		
-7.1	49.8		
24.7	609.8		
86.8	7533.2		
-22.7	515.6		
SUM =	1529.3	**S_d =**	39.1
		$t_{calculated}$ =	2.260

Table 3. Comparison of the manual vs. automated calibration curve preparation using the student's t-test determination for Compound B. At seven degrees of freedom for 95% confidence level the $t_{calculated}$ and t_{table} were determined to be 2.260 and 2.365, respectively. In this case, the $t_{calculated} < t_{table}$, thus the data are within the 95% confidence level.

Figure 12 displays a typical chromatogram at the LLQ (Lowest Limit of Quantification) for compounds A and B. The LLQ and HLQ (Highest Limit of Quanification) for the experiment were 10 ng/mL and 2500 ng/mL, respectively based on 50 uL of rat plasma. Both compounds A and B had retention times of 0.63 minutes and the internal standard has a retention time of 0.66. The solvent front eluted between 0.38 and 0.50 minutes for all compounds. The solvent front is the material from the vial that is not retained on the column and is seen in the figure as the baseline of each chromatogram. If the analytes were to elute at this particular time their signals would be reduced dramatically.

Signal to Noise Ratios (S/N) for the LLQ of compounds A and B were approximately 10/1. Compounds A and B peaks had 4200 and 9000 counts, respectively. The carryover for compounds A and B for this experiment were determined to be 0.08 % and 0.13 %, respectively. Determination of the carryover was performed by analyzing a 2500 ng/mL (HLQ) and immediately injecting a blank sample with internal standard added. The calculation of the internal ratio of analyte to internal standard will be determined for both the HLQ and the blank. The internal ratio of the blank divided by the internal ratio of the HLQ multiplied by 100 will give the percentage of carryover.

The sequence of the run was performed by first analyzing the replicates of standards following by the unknowns, and then running the second set of standards.

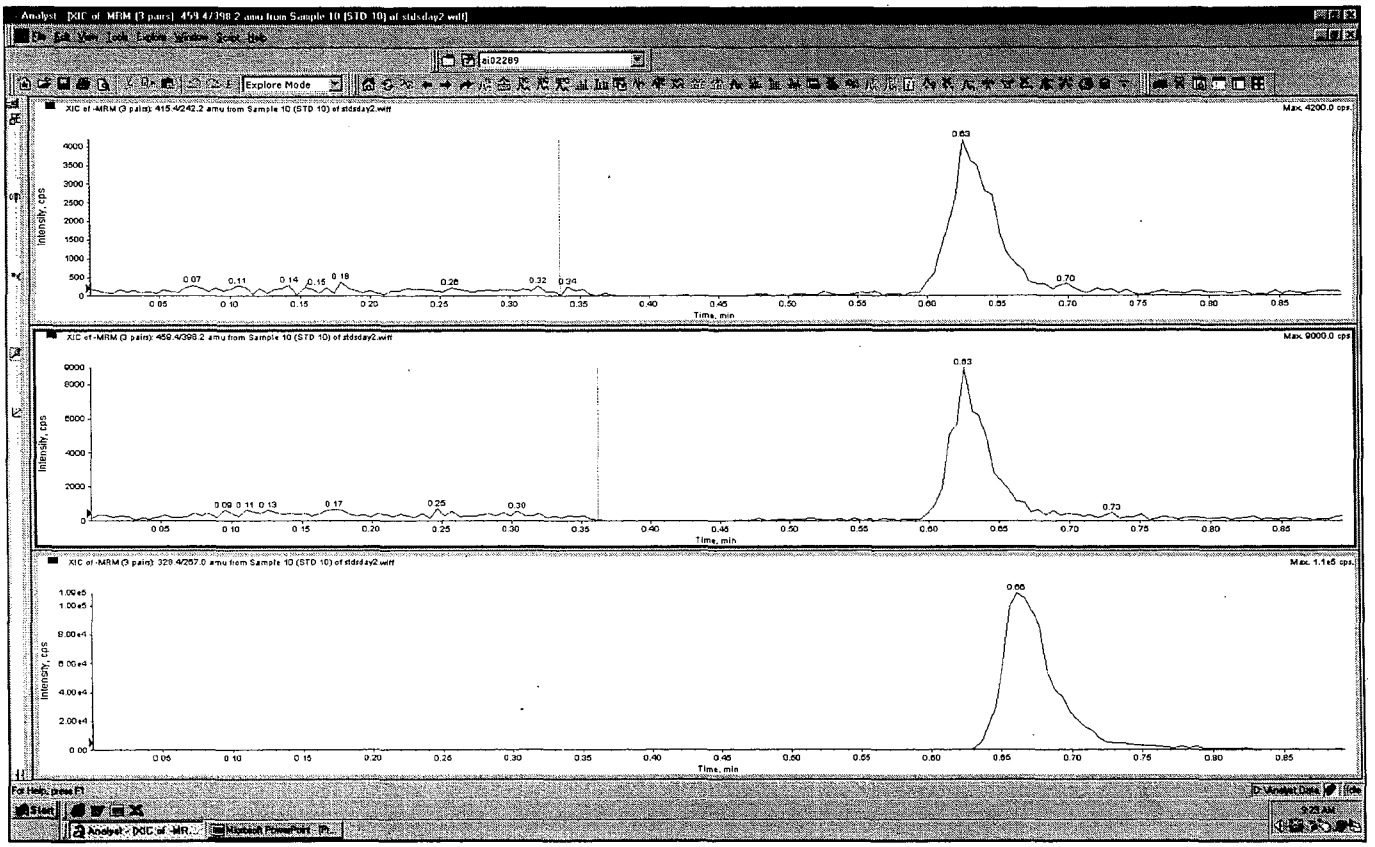

Figure 12. Chromatogram at the LLQ (10 ng/mL) of Compounds A and B in rat plasma. Compounds A, B, and IS are the top, middle and bottom chromatograms, respectively.

Results of the Three Day Validation

The standard curves were prepared in duplicate each day by utilizing the multiprobe with the same preparation as in Table 1, however, only compound A was used. The first working solution was prepared by adding 40 uL of the 1.00 mg/mL Compound A to 360 uL of 50/50 acetonitrile/water. The 1.00 mg/mL solution of compound A for the standard was from a separate weighing than the quality control 1.00 mg/mL stock solution. The quality control samples (QCs) were prepared each day by the multiprobe at four levels (10 ng/mL, 25 ng/mL, 1000 ng/mL, and 2500 ng/mL). Each level of the quality control samples was prepared in six replicates, therefore, 24 QC samples were generated per run. Blank samples were also used to confirm minimal contamination and carryover levels. The preparation of the QC samples is in Table 4.

QC preparation concentration (ng/mL)	Working Std used	volume of solution (uL)	rat plasma volume (uL)
2500	100	15	585
1000	20	30	570
25	2	7.5	592.5
10	0.2	30	570

Table 4. Quality Control sample preparation. After the final concentrations are prepared, the multiprobe aliquoted 50 uL of each level into six replicates.

One hundred μL of internal standard (IS) in acetonitrile was added to the tubes containing 50 uL of plasma and vortex mixed. All quality controls samples, standards, and blanks were centrifuged for 10 minutes at 4000 rpm. Each run consisted of 24 QC samples, 16 standards and 2 blank plasma samples. One of the

blanks contained the internal standard and the other was protein precipitated with 100 % acetonitrile. The results of the standards for the three runs are in Table 5. The three runs were combined and statistics including the average mean, standard deviation for all replicates, average bias, average %CV, within run precision, and between run precision were calculated. The above results were calculated in the following manner:

$$\textbf{Bias} = \frac{\text{Mean concentration X 100}}{\text{Nominal concentration}} - 100$$

$$\textbf{Average Bias} = \frac{\text{Bias}_{run1} + \text{Bias}_{run2} + \text{Bias}_{run3}}{\text{Number of Runs}}$$

$$\textbf{\% CV} = \frac{\text{Standard deviation of replicates}}{\text{Mean measured concentration}} * 100$$

$$\textbf{Average within-run precision} = \frac{CV_{run1} + Cv_{run2} + CV_{run3}}{\text{Number of runs}}$$

$$\textbf{Between run precision} = \frac{\text{S.D. determined from the within-run means X 100}}{\text{Average of within-runs means}}$$

The results of the QC samples analyzed are in Table 6. The statistical information for this validation is in Table 7.

Back-calculated calibration standards and curve parameters for the three day validation of Compound A

	Calibration standard nominal concentration (ng/mL)										
	10	**25**	**50**	**100**	**250**	**500**	**1000**	**2500**	**Slope**	**Intercept**	**r^2**
RUN 1	10.7	26.3	54.2	85.0	267.1	467.7	957.2	2584.1	0.0017	0.0027	0.99896
	11.0	26.6	48.0	88.5	254.0	454.8	996.0	2538.7			
RUN 2	9.8	35.2	43.5	89.0	267.2	479.0	970.9	2556.0	0.0017	0.002	0.99808
	10.3	32.3	40.6	85.0	228.9	478.5	921.5	2622.3			
RUN 3	10.7	22.4	45.4	82.9	244.7	464.1	928.4	2576	0.0013	-0.0013	0.99806
	12.5	28.6	50.5	93.5	282.1	505.6	913.7	2609			
Mean	10.8	28.6	47.0	87.3	257.3	474.9	947.9	2581.0	0.0016	0.0011	0.99837
Std.Dev.	0.9	4.6	4.9	3.8	18.9	17.6	32.2	31.4			
CV%	8.2	16.1	10.4	4.4	7.3	3.7	3.4	1.2			
% of Nominal	108.4	114.3	94.1	87.3	102.9	95	94.8	103.2			
# of reps.	6	6	6	6	6	6	6	6			

Table 5. Back calculated standards for all runs of the three day validation. Each level for each run had two replicates of standards.

	Nominal Conc. (ng/mL)			
	10	**25**	**1000**	**2500**
RUN 1	9.3	20.8	1022.5	2658.4
	8.9	25.8	1091.9	2931.3
	8.6	22.7	985.3	2849
	9.1	25.7	959.7	2736.6
	8.6	24.3	929	2617.5
	8.3	22	889.5	2620.7
Mean	8.8	23.5	979.6	2735.6
S.D.	0.4	2.1	71.5	129.8
CV %	**4.2**	**8.7**	**7.3**	**4.7**
Bias %	**-12**	**-5.8**	**-2**	**9.4**
Total number of rep. per run	6	6	6	6
RUN 2	7.9	24.9	1052.9	2837.6
	9.7	23.8	1115.7	2537.5
	10.6	24.2	1012.3	2748.1
	9.4	24.9	999.3	2849
	9.3	27.9	1114.9	2530.4
	8.5	22.3	953.6	2599.7
Mean	9.2	24.7	1041.5	2683.7
S.D.	0.9	1.8	65.4	146.4
CV %	**10.1**	**7.4**	**6.3**	**5.5**
Bias %	**-7.8**	**-1.3**	**4.1**	**7.3**
Total number of rep. per run	6	6	6	6
RUN 3	8.7	22.9	862.5	2364.5
	11.3	27.3	832.4	2845.9
	10.9	26.8	1018.0	2679.3
	10.7	26.2	1031.6	2566.8
	11.6	25.0	1015.2	2655.7
	12.9	25.8	1027.3	2633.0
Mean	11.0	25.7	964.5	2624.2
S.D.	1.4	1.6	91.3	157.5
CV %	**12.6**	**6.1**	**9.5**	**6.0**
Bias %	**10.2**	**2.8**	**-3.6**	**5.0**
Total number of rep. per run	6	6	6	6

Table 6. Results and statistics of quality control samples in rat plasma in the three runs

	Nominal Conc. (ng/mL) 10	Nominal Conc. (ng/mL) 25	Nominal Conc. (ng/mL) 1000	Nominal Conc. (ng/mL) 2500
Mean of all quality control samples	9.7	24.6	995.2	2681.2
S.D.	1.4	1.9	79.9	144.0
CV%	14.0	7.9	8.0	5.4
Average Bias %	-3.2	-1.5	-0.5	7.2
Total # reps	18.0	18.0	18.0	118.0
Average Within Run Precision %	10.2	7.4	7.7	5.4
Between Run Precision (%)	11.4	3.1	2.6	-1.0

Table 7. Overall results from the three runs of the quality control samples of Compound A.

Conclusions

The manual versus automated methods were analyzed from 10.0 ng/mL to 2500 ng/mL for compounds A and B. The t statistic from the student's t test for compounds A and B were 2.246 and 2.260, respectively. The 95 % confidence level (7 degrees of freedom) student's t_{table} was 2.365. Since the experimental results were less than the reference value the two methods are said to be acceptable for both compounds A and B. The student's t third case was used because two different methods were used to make single measurements on different samples.

For compounds A and B, the standard curves for the automated technique were slightly lower than the manual method. This may have been due to small droplets from the wash station, which may have added a small amount

of solvent and caused a slight dilution to all levels of standards. No wash station was used for the standards when they were prepared manually. Another type of systematic error may have been encountered by the accuracy of the pipet used by the analyst. Calibration of the pipet is accurate only at an error of +/- 3%. The repeatability of duplicate measurements of the standard curve appeared to be slightly better for the automated method than for the manual curve derived from manually prepared samples. A few factors that may have contributed to the lower regression for the manual method are human error, the use of different pipets, and evaporation of the working solutions.

One of the most important advantages to using robotics over manual sample preparation is that the amount time to prepare standards and samples is cut in half. The analyst has more time to perform other important tasks in the laboratory.

The three run validation had an LLQ of 10 ng/mL and an HLQ (highest limit of quantification) of 2500 ng/mL. The three day validation for compound A was performed using the multiprobe since it was shown that both the manual and automated methods were not distinctly different. The % cv, average % cv, bias, and average bias for all four levels of quality control samples must be within 15% including within and between run precision in order to meet the Good Laboratory Practices guidelines. For this study, all of the levels of quality control samples did meet these criteria. There were no standards or quality control samples removed from the entire three day validation.

A rapid, sensitive, and selective LC/MS/MS method has been developed and successfully validated for the determination of compound A in this experiment. This technique may be used for many other programs or types of new chemical entities since it is a general detection and quantification procedure with high selectivity. The key points that must be met to use this technique is the sensitivity for formation of a molecular ion by the compound of interest. This automated method is applicable for the analysis of other small organic molecules.

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Biography

Richard James Grater was born on May 26,1969 in Trooper, PA (just outside of Valley Forge National Park). His parent's names are William and Ann Marie Grater. He graduated from Bloomsburg with his Bachelors of Science Degree in Chemistry in May 1993. He became employed at Tektagen Inc. as an analyst in October 1993 where he did most of his work on protein assays. He joined GlaxoSmithKline Pharmaceuticals (formally known as SmithKline Beecham Pharmaceuticals) as a scientist in May 1996 and began working on the LC/MS/MS. He has been pursuing a Masters of Science Degree in Pharmaceutical Chemistry part time since August 1997. On a personal note, he enjoys playing football, fishing, mountain biking, and swimming in his spare time.

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